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Development and validation of a normal-phase high-performance thin layer chromatographic method for the analysis of sulfamethoxazole and trimethoprim in co-trimoxazole tablets

D.H. Shewiyo[a,b,c], E. Kaale[b], P.G. Risha[b], B. Dejaegher[c], J. Smeyers–Verbeke[c], Y. Vander Heyden[c,*]

[a] Directorate of Laboratory Services, Tanzania Food and Drugs Authority, P.O. Box 77150, Dar es Salaam, Tanzania
[b] School of Pharmacy, Muhimbili University of Health and Allied Sciences, P.O. Box 65526, Dar es Salaam, Tanzania
[c] Analytical Chemistry and Pharmaceutical Technology, Pharmaceutical Institute, Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, 1090 Brussels, Belgium

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ABSTRACT

Pneumocystis carinii pneumonia (PCP) is often the ultimate mortal cause for immunocompromised individuals, such as HIV/AIDS patients. Currently, the most effective medicine for treatment and prophylaxis is co-trimoxazole, a synergistic combination of sulfamethoxazole (SMX) and trimethoprim (TMP). In order to ensure a continued availability of high quality co-trimoxazole tablets within resource-limited countries, Medicines Regulatory Authorities must perform quality control of these products. However, most pharmacopoeial methods are based on high-performance liquid chromatographic (HPLC) methods. Because of the lack of equipment, the Tanzania Food and Drugs Authority (TFDA) laboratory decided to develop and validate an alternative method of analysis based on the TLC technique with densitometric detection, for the routine quality control of co-trimoxazole tablets. SMX and TMP were separated on glass-backed silica gel 60 F_{254} plates in a high-performance thin layer chromatograph (HPTLC). The mobile phase was comprised of toluene, ethylacetate and methanol (50:28.5:21.5, v:v:v). Detection wavelength was 254 nm. The R_f values were 0.30 and 0.61 for TMP and SMX, respectively. This method was validated for linearity, precision, trueness, specificity and robustness. Cochran's criterion test indicated homoscedasticity of variances for the calibration data. The F-tests for lack-of-fit indicated that straight lines were adequate to describe the relationship between spot areas and concentrations for each compound. The percentage relative standard deviations for repeatability and time-different precisions were 0.98 and 1.32, and 0.83 and 1.64 for SMX and TMP, respectively. Percentage recovery values were 99.00% ± 1.83 and 99.66% ± 1.21 for SMX and TMP, respectively. The method was found to be robust and was then successfully applied to analyze co-trimoxazole tablet samples.

1. Introduction

One of the most common opportunistic infections in HIV/AIDS patients is pneumocystis carinii pneumonia (PCP) caused by *Pneumocystis jirovecii*, which mostly attacks the lungs. It is often the ultimate mortal cause for immunocompromised individuals. Currently, the most effective treatment is co-trimoxazole, a synergistic combination of sulfamethoxazole and trimethoprim in a ratio of 5:1 [1–3]. Chemically SMX is 4-amino-N-(5-methyl-3-isoxazolyl) benzene sulfonamide and TMP 5-(3,4,5-trimethoxybenzyl) pyrimidine-2,4-diamine (Fig. 1). Co-trimoxazole is lifesaving, simple and provides inexpensive intervention against PCP.

In an effort to facilitate adoption of co-trimoxazole prophylaxis against PCP in resource-limited countries, the World Health Organization (WHO) has developed guidelines providing global technical and operational recommendations for the use of co-trimoxazole prophylaxis in HIV-exposed children, children living with HIV, and adolescents and adults living with HIV in the context of scaling up HIV care [2]. It is therefore imperative for resource-limited countries to ensure that high quality co-trimoxazole products are continuously available in their markets for effective implementation of these recommendations.

The Tanzania Food and Drugs Authority (TFDA), a regulatory body under the Ministry of Health and Social Welfare, is implementing a two tier quality assurance program where, at the ports of entry, inspectors are trained and provided with a Minilab® kit for screening selected medicines in tier one [4]. The Minilab® kit is a non-laboratory-based Thin Layer Chromatography (TLC) testing kit. It can be used as a stand-alone testing site in remote areas

* Corresponding author. Tel.: +32 2 477 47 34; fax: +32 2 477 47 35.
E-mail address: yvanvdh@vub.ac.be (Y. Vander Heyden).

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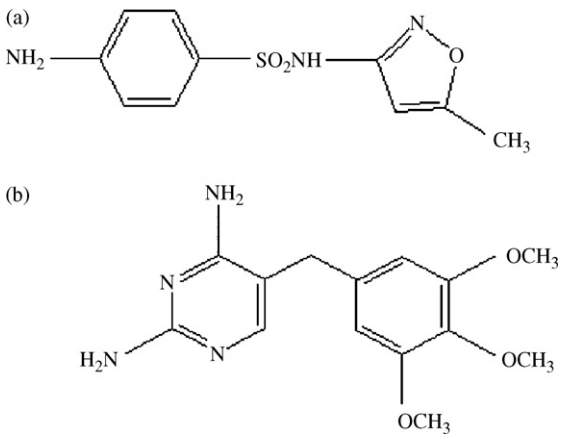

Fig. 1. Chemical structures of (a) sulfamethoxazole and (b) trimethoprim.

with minimal facilities. The tier two site is the central TFDA laboratory which performs full pharmacopoeial monograph testing to determine compliance to legal standards for samples which failed the screening tests [4]. The analytical method currently used for the analysis of co-trimoxazole in this laboratory is the HPLC one described in the United States Pharmacopoeia (USP) [5]. However, the lack of equipment and the long analysis time significantly affect timely release of laboratory results for regulatory action. Therefore, there was a need for an alternative method.

Most of the methods reported in the literature to analyze co-trimoxazole in pharmaceutical dosage forms, as well as in biological fluids, use HPLC [6–13] or spectrophotometry [14,15]. The drawbacks of HPLC for the TFDA laboratory have been mentioned. A spectrophotometric method also was not favored since the aim was to maintain and strengthen the use of the TLC technique. To our knowledge, the only method revealed in the literature using high-performance thin layer chromatography (HPTLC)—densitometry for the analysis of co-trimoxazole is reported by Dhasan et al. [16], where the effect of cystone on the availability of SMX and TMP in rabbits was studied. The drugs were assayed from rabbit's plasma and the TLC plate was scanned at two wavelengths, i.e. 265 and 350 nm for SMX and TMP, respectively. To apply the method for the analysis of SMX and TMP in dosage form formulations this method requires modifications in sample pretreatment, as well as the use of only one single wavelength to reduce analysis time.

In this study, a method for the routine analysis of SMX and TMP in co-trimoxazole tablets, which is precise, true, robust, and quick, is developed and validated. This method is based on HPTLC with ultraviolet/visible (UV/vis) densitometry scanning at a single wavelength. It strengthens tier two of the quality assurance program at the main TFDA laboratory by utilizing the same organic solvents as with the Minilab® kit. It also sustains the benefits of TLC, where up to 20 samples simultaneously can be analyzed on one plate. This results in a fast analysis with less cost per run and limited mobile phase consumption. Repeated scanning can be done by changing the scanning conditions [17,18]. The method was validated for linearity, precision, trueness, specificity and robustness, as recommended [19,20].

2. Experimental and methodology

2.1. Materials, chemicals and equipment

Ethylacetate was procured from Applichem (Darmstadt, Germany), methanol from Merck (Darmstadt) and toluene from BDH (Poole, England). All were of analytical grade.

SMX and TMP reference standards were obtained from the WHO Collaborating Centre for Chemical Reference Substances (Stockholm, Sweden). The tablet formulation matrix without active ingredient was a gift from Shelys Pharmaceuticals (Dar es Salaam, Tanzania). The matrix contained microcrystalline cellulose, sodium starch glycolate, magnesium stearate and purified starch. Co-trimoxazole tablets from different manufacturers were bought from retail pharmacies in Dar es Salaam, Tanzania.

HPTLC glass plates pre-coated with silica gel 60 F_{254} (10 cm × 20 cm) were from Merck. Densitometry was carried out with a Camag TLC Scanner 3 (Muttenz, Switzerland) fitted with winCATS 1.4.0 planar chromatography manager software. Samples were applied on the HPTLC plates using the spray-on technique of Camag Linomat V under nitrogen gas flow, and plates were developed in a Camag 20 cm × 20 cm twin trough chamber.

2.2. Method development and validation

2.2.1. Method development

HPTLC plates were pre-washed with methanol using the ascending technique and dried in an oven at 105 °C for 5 min. Standard and sample volumes of 2 μL were applied on the HPTLC plates as 6 mm bands. Application positions were at least 15 mm from the sides and 10 mm from the bottom of the plates. Reference standards, sample and spiked tablet matrix solutions were made using methanol as solvent. Mobile phase components were freshly mixed and the development chamber was left to saturate with mobile phase vapour for 15 min before each run. Development of the plate was carried out by the ascending technique to a migration distance of 6 cm. Then the plates were dried on a hot plate which is supplied with the Minilab® kit.

Densitometric scanning was done in absorbance/reflectance mode at 254 nm using a deuterium lamp. The slit dimensions were set at 5 mm × 0.45 mm, the scanning speed at 20 mm/s, and the data resolution at 100 μm/step. The laboratory room was under air conditioning and always maintained at 22 ± 2 °C temperature and 55 ± 5% relative humidity.

The separation of the spots was based on the TLC screening test for co-trimoxazole tablets, described in the GPHF Minilab® kit [21], in which the mobile phase was composed of ethyl acetate and methanol (75:15, v:v). The result of this procedure was a tailing TMP spot, and an SMX spot showing incomplete resolution from the solvent front. Therefore, several combinations of the mobile phase components were tested. Further addition of a third organic component, toluene, to the mobile phase was tested at varying ratios, with the objective of achieving a reproducible optimal separation between the spots ($Rs \geq 1$) and migration of spots within R_f values between 0.2 and 0.8 [22].

2.2.2. Method validation

Prior to the method validation process, instrument precision was evaluated in terms of sample application, positioning of TLC scanner stage and scanning of the same spot, as described in [22]. A reference standard solution with 74 and 98 mg/L SMX and TMP, respectively, prepared in methanol was used for these tests. For the test on sample application, a 100 μL syringe was filled with standard solution, and fixed on the sample application machine, Linomat V. 2 μL of the solution was repeatedly applied on the plate to make seven bands. The plate was developed and scanned. For the test on positioning the scanner stage, the plate developed during the sample application evaluation, was used. The scanning of the same spot was repeated seven times while the scanner stage was repositioned each time. For the test on scanning the same spot, the scanning of a given spot was repeated seven times. For all tests, the percentages relative standard deviations (%RSD) of the spot areas for each test were calculated and compared to the stated limits [22].

2.2.3. Linearity of the calibration line

A stock standard solution containing 660.0 and 816.0 mg/L SMX and TMP, respectively, was prepared and serially diluted to obtain solutions with 26.4, 52.8, 79.2, 105.6 and 132.0 mg/L SMX and 32.64, 65.28, 97.92, 130.56 and 163.20 mg/L TMP. For each standard solution, three bands of 2 μL were applied on the HPTLC plate in a distribution pattern with one on the left, middle and right side of the plate, respectively. This procedure was repeated for three days.

Homoscedasticity of variances along the regression line was verified using Cochran's test [23,24]. Since the variances were considered homoscedastic (see Section 3) for both regression lines, the slope and the intercept with their 95% confidence intervals were calculated using ordinary least squares [24]. The correlation coefficient r for both regression lines was also given.

The linearity was evaluated visually from the plotted calibration data, and statistically by performing an F-test for lack-of-fit (LOF). The LOF test examines whether the chosen model is appropriate, i.e. in this study, whether the straight line adequately fits the data [24].

2.2.4. Precision

The repeatability (within-day precision) and time-different intermediate precision (between-days precision) were determined simultaneously in one experimental set up. Solutions containing 60.0, 75.0, and 90.0 mg/L SMX and 75.0, 90.0, and 108.0 mg/L TMP were prepared in tablet matrix using methanol as solvent. They represented 80%, 100% and 120% of the target concentrations of each compound. Each day, they were analyzed in triplicate and this was repeated for six consecutive days. Fresh calibration curves to estimate the percentage recoveries were measured daily. The matrix used is composed with the most commonly used excipients.

The repeatability (s_r^2), and the time-different intermediate precision ($s_{I(t)}^2$), at each concentration level were estimated from an ANOVA table and Eq. (3) [23]

$$s_{I(t)}^2 = s_r^2 + s_{\text{between}}^2 \tag{3}$$

where s_{between}^2 represents the between-days variance.

2.2.5. Trueness

Tablet matrix portions were spiked with drug components at 80%, 100%, and 120% of the target sample concentrations, and extracted with methanol with the method procedure to obtain solutions with expected final concentrations of 60.3, 75.0, and 90.1 mg/L SMX and 72.2, 90.4, and 108.7 mg/L TMP. Three 2 μL volumes of these solutions were applied on the plate, in a pattern described in Section 2.2.3. Solutions were prepared in triplicate and analyzed for three consecutive days. Fresh calibration curves were measured daily to calculate the concentration of drug per spot. Trueness for each spot, evaluated by means of the percentage recovery, was calculated as;

$$\%\ \text{recovery} = \frac{C_{\text{obs}}}{C_{\text{exp}}} \times 100 \tag{4}$$

where C_{obs} and C_{exp} are the observed and the expected concentrations per spot, respectively.

The overall mean % recovery for each compound was calculated as;

$$\text{Mean \% recovery} = \frac{R_{\text{tot}}}{n} \tag{5}$$

where R_{tot} is the sum of all % recoveries and n the number of observations.

2.2.6. Specificity

Solutions of tablet matrix without drug components, and tablet matrix spiked with the drugs components at the same ratio as in tablets formulations (5:1 SMX:TMP) were prepared in methanol. About 200 mg of tablets matrix powder which is about twice the amount of matrix in each tablet was dissolved in a 100-mL volumetric flask using methanol. This solution of tablet matrix without drug components was made at high excipient concentration to enable detection of any excipients' spots with similar R_f values as the drug components. The two solutions were analyzed on the same plate and the chromatograms recorded.

2.2.7. Robustness

Experimental design-based robustness testing was carried out and evaluated as described in [25], using a Placket–Burman (PB) design with eight experiments. Factors whose effects were screened included (A) the developing distance of spots, (B) the amount of methanol in the mobile phase, (C) the drying conditions applied to the HPTLC plate after development, and (D) spot band size (Table 1). These parameters were chosen from observations made during method development and own experience. The limits were set to represent typical errors encountered in the laboratory; or were the only available options (for types of drying conditions).

Since four factors were examined, three dummy columns were included in the 8-experiments PB design (Table 2). All factors were studied at two levels. The effects of these factors on the responses percentage recoveries of SMX and TMP, R_f values of SMX and TMP, and resolution between SMX and TMP were estimated as follows;

$$E_x = \frac{\sum Y(+)}{N/2} - \frac{\sum Y(-)}{N/2} \tag{6}$$

where E_x is the effect of factor x on the response Y, $\sum Y(+)$ and $\sum Y(-)$ are the sums of the (corrected) responses where x is at (+) and (−) levels, respectively, and N is the number of design experiments.

A solution of tablet matrix spiked with SMX and TMP was measured at each design experiment. Spiking was performed at 100% of the target sample concentrations, i.e. 75.0 mg/L SMX and 90.0 mg/L

Table 1
The four factors and their levels.

Factor	Levels		
	(−)	Nominal (0)	(+)
(A) Developing distance of the spots (cm)	5	6	7
(B) Amount of methanol in the mobile phase (toluene:ethylacetate:methanol) (mL/L)	160	215	270
(C) Drying conditions applied on the plate after development	Air	Hot plate	Hot plate
(D) Spot band size (mm)	5	6	7

Table 2
Plackett–Burman design to examine four factors (A–D) and three dummies (d_i) in 8-experiments.

Experiment	Factors							Responses				
	A	d_1	B	d_2	C	d_3	D	% Recovery		R_f value		Resolution
								SMX	TMP	SMX	TMP	TMP–SMX
1	+1	+1	+1	−1	+1	−1	−1	99.66	98.42	0.58	0.29	19.33
2	−1	+1	+1	+1	−1	+1	−1	98.50	98.71	0.57	0.29	18.67
3	−1	−1	+1	+1	+1	−1	+1	100.12	99.65	0.56	0.28	18.67
4	+1	−1	−1	+1	+1	+1	−1	100.87	98.11	0.68	0.27	27.33
5	−1	+1	−1	−1	+1	+1	+1	97.75	98.34	0.71	0.34	24.14
6	+1	−1	+1	−1	−1	+1	+1	99.40	100.32	0.64	0.33	20.12
7	+1	+1	−1	+1	−1	−1	+1	99.66	100.88	0.62	0.27	23.33
8	−1	−1	−1	−1	−1	−1	−1	100.00	101.72	0.55	0.36	12.67

Responses	Effects of factors							Critical effect
	A	d_1	B	d_2	C	d_3	D	$ME_{\alpha=0.05}$
% Recovery SMX	0.81	−0.15	−0.21	0.53	1.21	−0.59	−0.73	1.63
% Recovery TMP	−0.17	−0.49	−1.78	−0.56	−0.86	−0.36	1.30	2.25
R_f value SMX	0.033	0.058	−0.038	−0.038	0.012	0.013	0.072	0.101
R_f value TMP	−0.028	−0.013	−0.018	−0.003	−0.013	0.052	0.008	0.038
Resolution (SMX–TMP)	3.99	2.67	−3.67	2.07	1.67	2.94	4.07	7.41

TMP. For each design experiment, standard solutions for calibration curves were also measured to calculate the percentage recoveries.

Factor effects were evaluated using Dong's algorithm, described in [25]. The algorithm of Dong, calculates from an initial estimate of error on an effect, s_0 (Eq. (7)) a final estimation of a standard error, s_1 (Eq. (8))

$$s_0 = 1.5 \cdot \text{median} \left|E_x\right| \tag{7}$$

$$s_1 = \sqrt{m^{-1} \sum E_i^2} \quad \text{for all} \quad \left|E_i\right| \leq 2.5 s_0 \tag{8}$$

where E_i is an effect that in absolute value is smaller than or equal to $2.5s_0$, and m the number of such effects. Then, a critical effect called the margin of error, ME, is calculated as follows,

$$\text{ME} = t_{(1-\alpha/2 \cdot df)} \cdot s_i \tag{9}$$

where $df = m$ and $\alpha = 0.05$. Factor values that are in absolute value larger than or equal to ME are considered significant.

2.2.8. Analysis of co-trimoxazole tablet formulation samples

The developed method was used to determine the amounts of SMX and TMP in three marketed co-trimoxazole tablet formulations samples. The label claims in all samples were 400 mg SMX and 80 mg TMP.

For each formulation, ten tablets were accurately weighed and ground to fine powder. SMX and TMP were determined in different solutions. Amounts of powder equivalent to 30 mg SMX and 36 mg TMP were accurately weighed into separate 100.0-mL volumetric flasks, where 70 mL of methanol was added. The two flasks were sonicated for 10 min, diluted to volume with methanol and mixed well. 5.0 mL of each solution was diluted into separate 20.0-mL volumetric flask to obtain final solutions with approximately 75.0 mg/L for the SMX solution and 90.0 mg/L for the TMP solution, respectively. A three-points calibration covering the upper, middle and lower limit of the calibration range were used to estimate the amount of drug in the sample formulations. The plates were developed and scanned using the densitometer to obtain the peak areas. The analysis was performed in triplicate.

3. Results and discussions

3.1. Method development

Since the initially tested mobile phase [21], which was composed of ethyl acetate and methanol (75:15, v:v) resulted in a tailing TMP spot and an SMX spot showing incomplete separation from the solvent front, several other combinations of the same mobile phase components were tested. Such combinations included ethyl acetate: methanol (50:50, v:v), (25:75, v:v), (90:10, v:v), (60:40, v:v) and (95:5, v:v). However, since these binary mobile phases did not lead to the aimed result, i.e. well-resolved spots ($Rs \geq 1$) with R_f values between 0.2 and 0.8, ternary mobile phases were evaluated. Toluene was introduced as a third component into the mobile phase, and it was observed to slow down the movement of SMX, separating it from the solvent front. Tested ternary mobile phase compositions included the combinations of toluene, ethyl acetate and methanol (60:25:15, v:v:v), (50:30:20, v:v:v), and (45:30:15, v:v:v). Finally, a mobile phase with a combination of toluene, ethylacetate and methanol (50:28.5:21.5, v:v:v) gave compact, symmetrical, well-resolved spots with R_f values of 0.30 and 0.61 for TMP and SMX, respectively. R_f values between 0.2 and 0.8, i.e. situations which are away from the spotting position and the solvent front, have been reported to have high R_f-value reproducibility and are hence considered appropriate for quantification of compounds in HPTLC methods [22]. The development was done for 6 cm on the plate and the development time was 17 min. The development chamber was saturated for about 20 min and after development, drying of the plates was done using a hot plate. Simultaneous detection of SMX and TMP was performed at 254 nm since both compounds are well known to exhibit sufficient ultraviolet absorption at this wavelength.

3.2. Instrument precision

The results for repeatability of sample application showed that the %RSD were 0.85% and 0.93% for SMX and TMP, respectively. These were within the stated limit, i.e. %RSD < 1.0% [22].

The results for position of the TLC scanner stage were %RSD 0.87% and 1.25% for SMX and TMP, respectively, complying with the stated limit (%RSD < 3.0) [22]. For the test on the ability of the instrument to scan the same spots repeatedly, it is stated that the % RSD of the spot areas should not be more than 2.0%. The results were

0.81% and 1.02% for SMX and TMP, respectively, hence, complying with the limit [22].

3.3. Linearity of the calibration line

Before performing regression, the homoscedasticity of the calibration standards was verified using a Cochran's test. For SMX, the test statistic, C_{calc}, was found to be 0.261, and for TMP, it was 0.336. These calculated test-statistic values were smaller than the critical value, $C_{tab(\alpha=0.05;\ k=5,\ n=9)} = 0.439$. Thus, the variances of the calibration standards were considered to be homoscedastic. Therefore, ordinary least squares were used to estimate the regression lines.

The equations of the calibrations lines were $Area_{SMX} = 8.66\ C_{SMX}(ng/spot) + 490.75$ and $Area_{TMP} = 5.40C_{TMP}(ng/spot) + 66.34$ for SMX and TMP, respectively. For SMX, the values for the slope and the intercept with their confidence limits at 95% level were 8.66 ± 0.16 and 490.75 ± 60.70, respectively. The values of these parameters for TMP were 5.40 ± 0.06 and 66.34 ± 27.90, respectively. The correlation coefficients, r, were 0.9997 and 0.9999 for the SMX and the TMP calibration curves, respectively.

Visual observation of the calibration curves showed that the lines were straight. The lack-of-fit test result for the SMX calibration data was $F_{calc} = 0.360$ and for TMP, it was $F_{calc} = 0.156$. These values were smaller than the critical value, $F_{tab(\alpha=0.05;\ df_1=3,\ df_2=40)} = 2.839$. This means that straight lines were considered adequate to describe the relationships between the spot areas and the concentrations (weight per spot) for each compound.

From the above it is observed that though the straight line model is correct for the considered calibration ranges, the intercept of the calibration lines is significantly different from zero. We also observed this for the HPTLC analysis of other compounds. A possible explanation is that intrinsically this kind of methods shows saturation on the spots and therefore produces curved calibrations. However, at narrow range, as here is the case, linearity can fit the calibration responses but resulting in an intercept deviating from zero. Consequences of the above are that a one-point calibration does not result in a proper estimation of a sample concentration and that at least two standards in the observed linear range need to be measured for calibration purposes.

3.4. Precision

The repeatability (within-days precision) expressed as percentage relative standard deviations (%RSD) for the SMX concentration at the 80%, 100%, and 120% levels were 0.72, 0.85 and 1.38, respectively, and the time-different intermediate precision (between-days precision) %RSD values were 1.40, 0.95 and 1.74, respectively. The %RSD values for the TMP concentration at the same concentration levels were 0.75, 0.97 and 0.77, respectively, for repeatability, and 1.80, 1.57 and 1.54, respectively, for time-different intermediate precision. The pooled repeatability precisions were 0.98 and 0.83 for the SMX and the TMP concentrations, respectively, and the pooled time-different intermediate precisions were 1.32 and 1.64, respectively.

All the values for the repeatability and the time-different intermediate precisions were found to be comparable to HPLC methods as those described in [6], where a micellar and a reversed-phase liquid chromatography method for the determination of SMX and TMP in commercial pharmaceutical products, are compared. In both cases the percentage relative standard deviations are well below 2%. In conclusion, our %RSDs were considered acceptable.

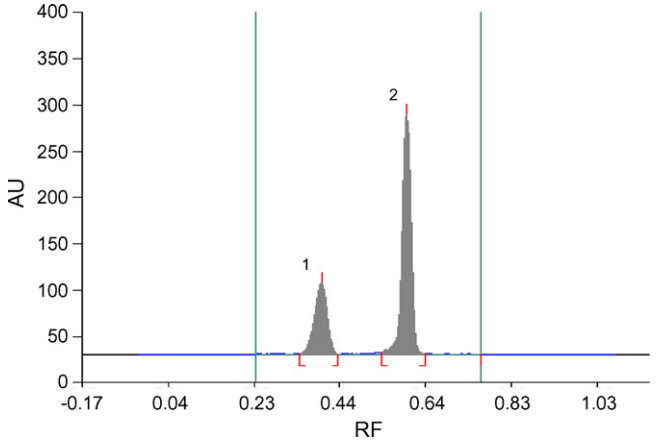

Fig. 2. Chromatogram of tablet matrix solution spiked with trimethoprim (1) and sulfamethoxazole (2).

3.5. Trueness

The recovery results obtained for SMX at the 80%, 100% and 120% concentration levels were $99.00\% \pm 1.64$, $100.15\% \pm 2.12$ and $100.27\% \pm 1.55$, respectively. For TMP, they were $99.95\% \pm 1.11$, $99.43\% \pm 1.47$ and $99.61\% \pm 1.11$, respectively. The ranges of the % recovery values covering all concentration levels for each compound were 96.56–102.93% for SMX and 97.46–101.95% for TMP. The overall mean recoveries for SMX and TMP were $99.00\% \pm 1.83$ and $99.66\% \pm 1.21$, respectively. Thus, the method was considered to have an acceptable recovery.

3.6. Specificity

The chromatogram of the solution of tablet matrix which was not spiked with SMX and TMP did not show any spot, while the chromatogram of the solution of tablet matrix spiked with SMX and TMP showed clear, compact and well-separated peaks of SMX and TMP (Fig. 2). Moreover, from Fig. 2, it was seen that no other peaks eluted besides the two active compounds. The method was therefore considered specific.

3.7. Robustness

The factor effects were calculated for each response (Eq. (6)), and presented in Table 2. Using Eqs. (7–9), the margin of errors or the critical effects were calculated for each response. All factor effects on a given response that are in absolute value larger than or equal to the critical effect for the considered response are considered significant.

Table 3
Results of analysis of marketed formulations.

Product name	Manufacturer	Batch No.	Percentage amount found	
			SMX	TMP
Shetrim®	Shelys Pharmaceuticals Ltd, Tanzania	F020	98.6 ± 1.6	101.6 ± 2.1
Altran®	Alpa Laboratories Ltd, India	TV-1783	97.2 ± 2.5	94.1 ± 3.7
Co-trimoxazole	North China Pharmaceutical Ltd, China	0803125	100.0 ± 2.4	101.9 ± 3.1

All absolute factor effects on the responses % recovery of SMX and of TMP, which represent the quantitative aspects of the method, were found to be smaller than the corresponding critical effects, i.e. $ME_{\alpha=0.05} = 1.63$ for SMX and 2.25 for TMP. Since no significant factor effects were found for these quantitative responses, the method was considered robust.

All absolute factor effects on the qualitative response R_f values of SMX were smaller than the critical effect, $ME_{\alpha=0.05} = 0.101$. On the other hand, on the response R_f values of TMP, one dummy, d_3 was considered to have a significant effect, since its absolute value, 0.052, was larger than the critical effect, $ME_{\alpha=0.05} = 0.038$. Since it concerns a dummy factor, it is considered non-significant by definition and it is further ignored. Moreover, since one is working at $\alpha = 0.05$, statistically one considers a non-significant effect as significant in one case out of twenty, which probably is the case here.

For the separation quality related response resolution between the SMX and the TMP spots, the absolute factor effects were smaller than the critical effect, $ME_{\alpha=0.05} = 7.410$. Thus, no significant effects were indicated for the response resolution.

3.8. Results of analysis of co-trimoxazole tablets formulations

Analysis of real samples of marketed co-trimoxazole tablet formulations was performed using this method. The percentage amounts for SMX and TMP were calculated from the label claims, i.e. 400 mg SMX and 80 mg TMP. These are reported with their standard deviations in Table 3. Percentage amounts of SMX in all formulations were found to range between 97.2% and 100% and from 94.1% to 101.9% for the TMP. They all complied with the prescribed limits by the USP (90.0–110.0%), in which the assay is performed using HPLC [5].

4. Conclusion

A quick, precise and true method in normal-phase HPTLC for routine analysis of SMX and TMP in co-trimoxazole tablet formulations has been developed and validated. It uses the chemicals and solvents that are also applied in the Minilab® kit, hence strengthening the ongoing quality assurance program for medicines at TFDA. Up to 20 samples can be analyzed on one plate in a short time, therefore saves time and cost per run. While it takes about 15–17 min to develop one plate that may contain as many as 20 samples, it takes about 8 min to run one injection on HPLC (about 2:40 h for 20 samples). If we consider sample preparation until densitometric evaluation for the above plate, it takes about 45 min to 1 h, whereas it takes more than 3 h for the HPLC method. In terms of cost, the extra time acquired in HPTLC is used for analysis of more samples. Again, while the HPLC method consumes about 250 mL of mobile phase for the above samples, HPTLC utilizes less than 20 mL.

With this method, regulatory decisions are timely facilitated, which helps to have a continuous availability of high quality co-trimoxazole tablets in the Tanzanian market for the treatment and prophylaxis of various infections, including *Pneumocystis jirovecii* (PCP), in HIV/AIDS patients.

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References

[1] T. Johnson, I.A. Khan, M.A. Avery, J. Grant, S.R. Meshnick, Antimicrob. Agents Chemother. 42 (1998) 1454.
[2] Guidelines on Co-trimoxazole prophylaxis for HIV-related infections among children, adolescents and adults in resource limited settings, Recommendations for a public health approach, HIV/AIDS programme, WHO, Geneva, 2006.
[3] B.T. Mmbaga, G. Kinabo, M. Swai, J. F. Shao, J. Mchele, M. Henderikx, B. Mulder, E. Houpt, J. Tolboom, W. Schimana, Positive effects of Co-trimoxazole prophylaxis in HIV Infected children in Kilimanjaro Tanzania, a poster presentation available at (http://www.retroconference.org/2007/PDFs/705.pdf), accessed on 5th February 2009.
[4] P.G. Risha, Z. Msuya, M. Clark, K. Johnson, M.N. Sigonda, T. Layloff, Health Policy 87 (2008) 217.
[5] The United States Pharmacopoeia 24, National Formulary 19, United States Pharmacopoeia Convention, Rockville MD, USA, 1995.
[6] A.U. Kulikov, A.G. Verushkin, L.P. Loginova, Chromatographia 61 (2005) 455.
[7] H. Amini, A. Ahmadiani, J. Pharm. Biomed. Anal. 43 (2007) 1146.
[8] N.A. Épshtein, Pharm. Chem. J. 36 (2002) 675.
[9] A.V. Pereira, Q.B. Cass, J. Chromatogr. B. 826 (2005) 139.
[10] R. Gochin, I. Kunfer, J.M. Haigh, J. Chromatogr. 223 (1981) 139.
[11] M. Chair, H.J. Nelis, P. Leger, P. Sorgeloos, A.P. De Leenheer, Antimicrob. Agents Chemother. 40 (1996) 1649.
[12] J. Chakwenya, J. Lakrittz, J. Tyler, W.H. Fales, M. James-Kracke, K. Smith, J. Holle, J. Vet. Pharmacol. Ther. 25 (2002) 321.
[13] M.Y. Haller, S.R. Muller, C.S. McArdell, A.C. Alder, M.J.F. Suter, J. Chromatogr. A 952 (2002) 111.
[14] S. Balyejjusaa, R.O. Adomeband, D. Musok, Afr. Health Sci. 2 (2002) 56.
[15] J.J. Berzas Nevado, J.M. Lemus Gallego, G. Castafieda Pefialvo, J. Anal. Chem. 342 (1992) 723.
[16] P.B. Dhasan, M. Tripathi, R. Sundaram, M.V. Venkataranganna, S. Gopumadhavan, K.S. Kulkarni, Indian Drugs 38 (2001) 526.
[17] J. Ali, N. Ali, Y. Sultana, S. Baboota, S. Faiyaz, Acta Chromatogr. 18 (2007) 168.
[18] H. Kalasz, M. Bathori, in: K. Valko (Ed.), Handbook of Analytical Separations, 1, Elsevier, Amsterdam, 2000, p. 439.
[19] Guidelines prepared within the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human use (ICH), Validation of Analytical Procedures: Text and Methodology, Q2(R1). http://www.ich.org/, 2005, 1–13 (accessed on January 26th 2009).
[20] W. Funk, V. Dammann, G. Donnevert, Quality Assurance in Analytical Chemistry, second ed., Wiley-VCH, Weinhein, 2007.
[21] R.W.O. Jahnke, A concise Quality Control Guide on Essential Drugs; Volume II Thin layer Chromatography, in: Global Pharma Health Fund, (GPHF) Franfurt, Germany, 2001.
[22] J.C. Touchstone, Practice of Thin Layer Chromatography, third ed., John Wiley & Sons, New York, 1992.
[23] G.E.P. Box, W.G. Hunter, J.S. Hunter, An Introduction to Design, Data Analysis and Model Building, J. Wiley, New York, 1978.
[24] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers–Verbeke, Handbook of Chemometrics and Qualimetrics: Part A, Elsevier, Amsterdam, 1997, pp. 121, 189.
[25] Y. Vander Heyden, A. Nijhuis, J. Smeyers–Verbeke, B.G.M. Vandeginste, D.L. Massart, J. Pharm. Biomed. Anal. 24 (2001) 723.